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RESEARCH PAPER

Potential use of *Zataria multiflora* essential oil to control postharvest *Aspergillus flavus* fruit rot of strawberry

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إمكانية إستخدام الزيت العطري المستخرج من *Zataria multiflora* للسيطرة على تعفن فاكهة الفراولة بعد الحصاد

ماجدة بنت سعيد بن حمد الحضرمية[1] و زينب البلوشية[1] و عيسى بن هاشل المهمولي[1] و عبدالله بن محمد السعدي[1] و ريثيناسامي فيلازهاهن[1]*

Abstract. Postharvest fruit rot is a major problem in strawberry production chain worldwide. *Aspergillus* sp. is one of the major fungi associated with fruit rot of strawberry. In this study, an *Aspergillus flavus* was isolated from a rotten strawberry fruit. Based on the nucleotide sequence analysis of the internal transcribed spacer regions of rDNA, the fungus was confirmed as *A. flavus*. Pathogenicity of the isolated fungus was confirmed by artificially inoculating strawberry fruits under laboratory conditions. This strain was capable of producing aflatoxin B1 *in vitro* as determined by liquid chromatography-mass spectrometry analysis. Postharvest dip treatment of mature strawberry fruits with *Zataria multiflora* essential oil (ZEO) (0.1%) completely suppressed *A. flavus* infection and prevented rotting of fruits. The results of this study suggest that ZEO can be used as a sustainable and safe alternative to chemical fungicides for the control of *Aspergillus* fruit rot of strawberry.

Keywords: *Aspergillus flavus*, aflatoxin B1, essential oil, fruit rot, strawberry, *Zataria multiflora*

الملخص: تعد مشكلة تعفن ثمار الفاكهة مشكلة رئيسية في سلسلة إنتاج الفراولة في جميع أنحاء العالم. يعد فطر أسبرجيلس أحد الفطريات الرئيسية المرتبطة بتعفن ثمار الفراولة.في هذه الدراسة تم عزل عزلة من فطر أسبيرجيلس فليفس من ثمار فراولة فاسدة. اعتمادا على دراسة القواعد الوراثية للمنطقة المصانة ريبوسومي للحمض النووي تم تأكيد الفطر على أنه أسبيرجيلس فليفس. و تم تأكيد القدرة الإمراضية للفطر عن طريق التلويث اليدوي لثمار الفرولة تحت ظروف مخبرية. أظهرت الدراسة قدرة هذه السلالة على إنتاج الأفلاتوكسين (B١) في المختبر من خلال تحليل الكروماتوغرافيا السائلة الطيف الكتلي. كما أشارت الدراسة إلى أن غمس ثمار الفراولة الناضجة بعد الحصاد في الزيت العطري للزعتر *Zataria multiflora* (ZEO) بنسبة ٠,١ ٪ يكبح عدوى الفطر ويمنع تعفن الثمار تماما. تشير نتائج هذه الدراسة إلى أنه يمكن إستخدام الزيت العطري للزعتر ZEO بديلا آمنا ومستداما للمبيدات الفطرية وذلك لمكافحة تعفن ثمار الفراولة باسبيرجيلس.

الكلمات المفتاحية: أسبيرجيلس فليفس ، أفلاتوكسين B1 ، الزيت العطري ، تعفن الثمار ، فراولة ، *Zataria multiflora*

Introduction

Strawberry (*Fragaria* × *ananassa* Duch.) is one of the popular berries in the world. Strawberry fruits are preferred by consumers because of their attractive colour, aroma, taste and nutritional qualities (Bhat et al., 2015). It is one of the principal fruit crops cultivated in the greenhouses in Oman. Strawberry fruits are rich source of fiber and several phytochemicals, such as folic acid, vitamin C and other antioxidants (Chandler et al., 2012). It is being used as fresh fruit or as sliced and processed fruit (Chandler et al., 2012; Giampieri et al., 2012). These fruits are highly prone to attack by several fungal pathogens because of their succulent nature that can result in postharvest decay. The most common fungal pathogens that cause postharvest decay in strawberry fruits are *Botrytis cinerea, Rhizopus stolonifer, Mucor* spp., *Colletotrichum acutatum, Geotrichum candidum, Penicillium expansum, Cladosporium* spp., *Alternaria* spp. and *Aspergillus* spp. (Feliziani and Romanazzi, 2016; Ma et al., 2018; Palmer et al., 2019; Al-Rahbi et al., 2021). A few postharvest fungal pathogens of strawberry are known to produce foodborne mycotoxins (Hussein et al., 2020) and hence the quality of fruits after harvest is important for consumer's safety. For instance, *A. flavus* produces aflatoxin, a naturally occurring carcinogenic mycotoxin (Khlangwiset et al., 2011) and *Alternaria* spp. produce foodborne mycotoxins including alternariol, alternariol methyl ether, tentoxin, tenuazonic acid, altenuene and altertoxins (Escriva et al., 2017). Postharvest diseases of strawberry can be managed by avoiding mechanical damage to fruits by following safe handling practices, cold storage of fruits, storage of fruits under modified storage atmosphere, application of chemical fungicides, biocontrol agents and natural products like plant extracts and essential oils, and physical method like UV-C treatment (Feliziani and Romanazzi, 2016).

A. flavus is one of the most common postharvest fungal pathogens of fruits. Several studies reported the inhibitory activity of essential oils (EOs) from plants against *A. flavus* and aflatoxin production (Vilela et al., 2009; Kumar et al., 2010; Tian et al., 2012a; Kedia et al., 2014; Kedia et al., 2015; Kiran et al., 2016; Chaudhari et al., 2020). The EOs are volatile and odorous liquids extracted from plant tissues. The EOs pass through the cell wall of susceptible fungi because of their lipophilic nature and cause damage to the cytoplasmic membrane,

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coagulation of the cytoplasm and leakage of cellular macromolecules (Hyldgaard et al., 2012; Dwivedy et al., 2016). Gandomi et al. (2011) reported that *Zataria multiflora* EO induced morphological and structural changes in *A. flavus* including loss of turgidity, vacuolization of cytoplasm and deformation of hyphae. An increase in the generation of reactive oxygen species (ROS) and mitochondrial membrane potential and decrease in ATPase and dehydrogenase enzyme activities, and ergosterol content were reported in *A. flavus* due to the effect of *Anethum graveolens* (Dill) EO (Tian et al., 2012b). Chaudhari et al. (2020) demonstrated that Allspice (*Pimenta dioica*) EO caused damage to plasma membrane of *A. flavus* that resulted in the leakage of cellular ions. Al-Harrasi et al. (2021) recently reported the antifungal activity against *A. flavus* and aflatoxin B1-detoxification potential of *Zataria multiflora* (Lamiaceae) essential oil (ZEO). In this study, *A. flavus* was isolated from diseased strawberry fruits and tested for its pathogenicity and aflatoxigenic potential. Furthermore, the efficacy of ZEO in the control of *A. flavus*- induced postharvest fruit rot of strawberry was studied. This is the initial study in Oman describing isolation of an aflatoxigenic strain of *A. flavus* from rotted strawberry fruits as well as demonstrating the effectiveness of ZEO in the management of *A. flavus* fruit rot of strawberry.

Materials and Methods

Collection of Fruits

Strawberry fruits showing fruit rot symptoms were collected from a supermarket in Muscat, Sultanate of Oman and transferred to the laboratory in an ice box and used within 24 h after collection for isolation of fungus.

Isolation of Fungus

Diseased tissues (5×5 mm, approx.) from the fruits were cut with a sterile scalpel and surface-disinfected with NaOCl solution (1%) for 2 min, rinsed 2-3 times with sterile distilled water (SDW), blot-dried on sterile Whatman No. 1 filter paper and then placed on potato dextrose agar (PDA) medium (Oxoid Ltd., UK) in 9-cm diameter Petri dishes. The plates were sealed with parafilm and incubated at 27°C for 3-4 days. The pure culture of the fungus was obtained by single spore isolation method and the culture was maintained on PDA slants at 4°C.

Pathogenicity Test

Healthy strawberry fruits were surface sterilized with 70% ethanol for 5 s, rinsed twice with SDW and air-dried under aseptic condition. On the surface of each fruit, minute wounds were created by using a sterile inoculation needle. Twenty µl of spore suspension (1×10^6 spores/ml) of the fungal isolate prepared from a 5-day-old PDA culture was applied on the wounded site and incubated for 7-10 days at 25°C. Strawberry fruits applied with SDW served as control. Disease severity was measured using a 0-8 scale developed for gray mold of strawberry, where 0 indicated no disease and 8 indicated 87.6-100% of fruit decayed (Chen et al., 2018).

Aflatoxin B1 Production by Fungal Isolate

Agar disc (6 mm diameter) of the fungus taken from a 7-day-old PDA culture was transferred to 200 ml of sterile SMKY medium (200 g sucrose, 0.5 g magnesium sulfate, 0.3 g potassium nitrate, and 7.0 g yeast extract in 1 l of distilled water) (Tiwari et al., 2022) in a 500 ml-conical flask and incubated at 27°C. After 14 days of incubation the culture was filtered through Whatman No.1 filter paper and the culture filtrate was collected. In a sterile 1.5 ml centrifuge tube, 500 µl of the fungal culture filtrate and 500 µl of chloroform were added and vortexed for a few seconds. The tubes were then centrifuged at 12000 g at room temperature (25±2°C) and the chloroform layer was transferred to a new 1.5 ml tube and dried completely by using a water bath at 60°C. The residue was dissolved in 250 µl of analytical grade methanol and analyzed by liquid chromatography-mass spectrometry (LC-MS).

LC-MS Analysis

Analysis of Aflatoxin B1 was performed using an Agilent LC/MS/MS (Agilent Technologies, Inc., CA, USA) system, equipped with a high-performance auto sampler (G4226A), quaternary pump (G4204A), thermostated column compartment (G1316C) and Agilent 6460 Triple Quad Mass Spectrometer. The analyte separation was achieved using Symmetry C18; 5 µm, 3 mm × 150 mm column (Waters), with a mobile phase of water (A) and acetonitrile (B). Both the eluents were with 0.1% formic acid under gradient condition (eluent A 10-70% in 0-1 min, 70-95% in 1-2 min, hold at 95% for 4 min, 95-70% in 6-7.5 min, 70-10% in 7.5-8 min and hold at 10% for 1 min) with a flow rate of 0.3 ml per min. The column oven temperature was maintained at 45°C. Standard Aflatoxin B1 (Sigma-Aldrich, MO, USA) solution was used to optimize all MS parameters. The optimized MS parameter values were: gas temperature 300°C, gas flow 3 L min^{-1}, nebulizer pressure 50 PSI, sheath gas heater 375°C, sheath gas flow 10 L min^{-1}, capillary voltage 3500 V, scan range 100 to 3000 *m/z* and positive polarity. MS Data acquisition and analysis were performed using Agilent MassHunter Software.

Molecular Characterization of the Fungus

The genomic DNA extraction from the fungal isolate was carried out using the Plant/Fungi DNA Isolation Kit (Norgen Biotek Corp., Canada) as per the manufacturer's instructions. The amplification of the internal transcribed spacer (ITS) regions of the rDNA gene was done as suggested by Karthikeyan et al. (2009). Briefly, the PCR reaction was performed in a 25 µl reaction mixture consisting of a PuReTaq Ready-To-Go PCR bead

Figure 1. Growth of *Aspergillus flavus* isolated from decayed strawberry fruit on potato dextrose agar medium

(GE Healthcare, UK), 1 µl each of ITS4 and ITS5 primers (White et al., 1990) and 2 µl (100 ng) of the purified DNA and 21 µl of SDW. The thermal profile for PCR was as described by Halo et al. (2018). The amplification was confirmed by running 5 µl of the PCR product on 1% agarose gel in Tris-borate-EDTA (TBE) buffer followed by observation under UV light using a GeneFlash gel imaging system (Syngene, Cambridge, UK). The PCR product (~700 bp) was sequenced at Macrogen, Seoul, Korea. A homology search of the obtained nucleotide sequence was performed using the BLAST program (www.ncbi.nlm.nih.gov/BLAST) in order to identify the fungus.

Efficacy of ZEO in Controlling Aspergillus Fruit Rot of Strawberry

ZEO extracted previously (Al-Harrasi et al., 2021) was used in this study. Healthy strawberry fruits of uniform size and maturity were selected, and surface sterilized by dipping them in 1% NaOCl for 1 min and then washed twice in SDW. Then, they were dipped in ZEO (0.1% in distilled water) for 5 s and then air-dried in a laminar flow chamber for ~10 min. The fruits were then inoculated with *A. flavus* spore suspension as described earlier and incubated for 7 days at 25 °C. Strawberry fruits applied with SDW and inoculated with *A. flavus* served as control. Three fruits were used for each treatment and each treatment was replicated thrice.

Statistical Analysis

All experiments were performed in triplicate. The fruit rot disease severity data were expressed as mean ± standard deviation (SD).

Results and Discussion

In this study, *A. flavus* (isolate STR10) was isolated from strawberry fruits showing the symptoms of fruit rot and pure culture was obtained (Figure 1). To confirm the identity the fungus, the ITS region of rDNA was amplified by PCR using ITS4 and ITS5 primers and the amplified product was sequenced. BLAST analysis of the obtained nucleotide sequence showed 100% sequence similarity with that of *A. flavus* strain accessions MT635198, MT509715, MN893386, MN893385, MN533904 and MN533814 in the GenBank database. The nucleotide sequence of *A. flavus* isolate STR10 from this study was deposited in the GenBank with the accession number OL437469. The pathogenicity of the isolate was confirmed by artificial inoculation of strawberry fruits. The inoculated fruits exhibited the fluffy mycelial growth and symptoms of fruit rot 5-8 days after inoculation (disease severity score 6.4 ± 0.9) whereas the control fruits remained symptomless (disease severity score 0) (Figure. 2). *A. flavus* has been reported to cause postharvest rots in several fruits including grapes (Ghuffar et al., 2020), jujube (Singh and Sumbali, 2000), peach (Michailides and Thomidis, 2007), kiwi (Zhu et al., 2022) and

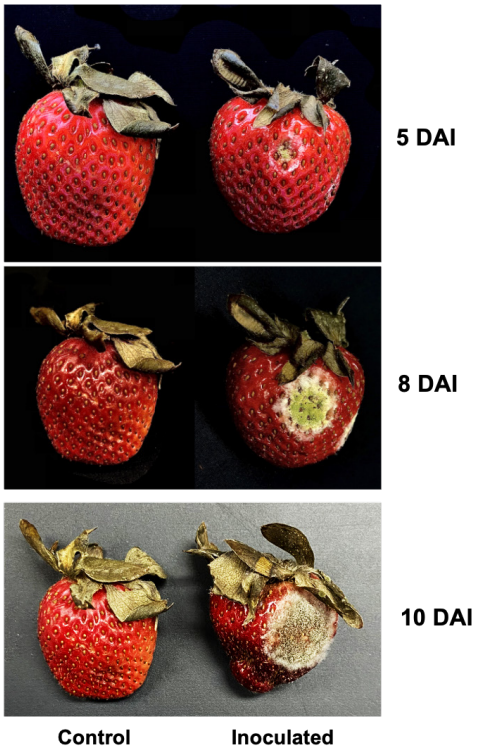

Figure 2. *Aspergillus flavus* infection on artificially inoculated strawberry fruits

Surface-disinfected strawberry fruits were inoculated with *A. flavus* spore suspension (1×10^6 spores/ml) and incubated at 25 °C. The pictures were taken 5, 8 and 10 days after inoculation (DAI).

lemon (Kotan et al., 2009). Several strains of *A. flavus* isolated from rotten fruits were shown to produce aflatoxins. The aflatoxins are carcinogenic, mutagenic and immunosuppressive compounds produced by *A. flavus* and *A. parasiticus* as secondary metabolites when the fungi grow on susceptible crops and food matrices under favourable conditions. Among the different forms of aflatoxins, aflatoxin B1 (AFB1) is considered as the most dangerous and toxic to humans and animals (Velazhahan, 2017). Saleem (2017) reported the production of aflatoxins by *A. flavus* and *A. parasiticus* from strawberry and the concentration of aflatoxins ranged from 23.6 to 71.1 ppb. Hussein et al. (2020) reported that *A. flavus* was recovered from 53.3%, of strawberry samples collected from Qena city, Egypt and the amount of aflatoxin produced by *A. flavus* was 3.5 ppb. The strain of *A. flavus,* STR10, isolated from infected strawberry fruits in this study was capable of producing aflatoxin B1 to a level of 4063 ± 993 ppb under in vitro conditions. Hence, control of *Aspergillus* fruit rot is of paramount importance to preserve the quality of harvested fruits for consumer's safety especially if the fruits are intended to be used as dry fruits. The natural products such as plant extracts and EOs have been suggested as an alternative to synthetic chemical fungicides for the control of postharvest diseases of fruits (Shao et al., 2013; Hosseini et al., 2020; Jahani et al., 2020; Raveau et al., 2020).

The results of the present study revealed that postharvest dip treatment of strawberry fruits with ZEO (0.1%) completely prevented *A. flavus* infection under laboratory conditions (Figure 3). The fungal mycelium covered 65-75% of the fruit surface within 5-7 days after inoculation in the untreated control (disease severity score 6.1 ± 0.8); whereas the ZEO-treated fruits showed no visible symptoms (disease severity score 0). The effectiveness of plant-derived EOs in controlling postharvest fruit rots have been reported in previous studies (Nikkhah et al., 2017; Hosseini et al., 2020). Garlic and rosemary EOs have been reported to control anthracnose (*Colletotrichum nymphaeae*) of strawberry (Hosseini et al., 2020). El-Mogy and Alsanius (2012) reported the suppression of *Botrytis cinerea* fruit rot of strawberry by Cassia EO. Alizadeh-Salteh et al. (2010) demonstrated the antifungal activity of Shiraz thyme EO against *Rhizopus stolonifer* the causal agent of *Rhizopus* rot of peach fruits. Mohammadi et al. (2015) demonstrated the effectiveness of combined application of chitosan with Z. *multiflora* or *Cinnamomum zeylanicum* EOs in the control of *B. cinerea* rot in strawberry. A few studies demonstrated the synergistic antimicrobial effect of EOs (Sharma and Sharma, 2011; Nguefack et al., 2012; Nikkhah et al., 2017). Nikkhah et al. (2017) demonstrated that pear fruits treated with cinnamon + rosemary + thyme or thyme + cinnamon EOs showed higher reduction in the lesion size of rot induced by *Botrytis cinerea* and *Penicillium expansum* than single EO treatments

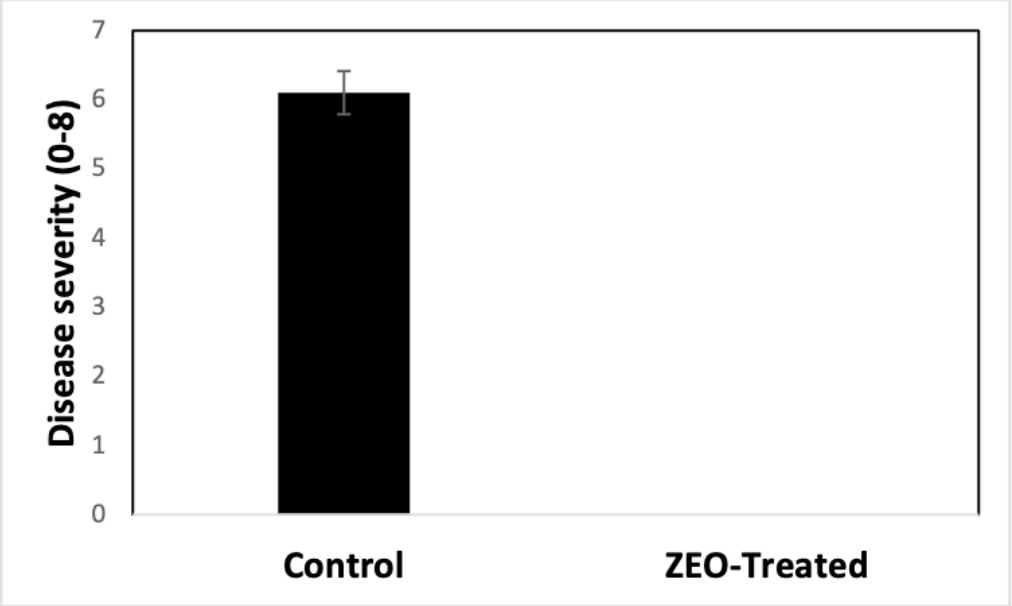

Figure 3. Effect of postharvest dip treatment of strawberry fruits with *Zataria multiflora* essential oil (ZEO) on *Aspergillus flavus* fruit rot

ZEO-treated or sterile distilled water-treated (control) strawberry fruits were inoculated with *A. flavus* spore suspension (1×10^6 spores/ml) and the disease severity was measured using a 0-8 scale after incubation at 25 °C for 7 days. Data are mean ± SD.

under laboratory conditions. A few essential oil-based commercial products such as Promax™, Sporan™, EcoPCOR and EcoTrol are being used as food preservatives to prevent fungal contamination (Dwivedy et al., 2016).

Conclusion

The results of this study suggest that ZEO can be used as a safe method for the control of *Aspergillus flavus* fruit rot of strawberry. The U.S. Food and Drug Administration (FDA) listed a number of EOs under "Generally Recognized as Safe (GRAS)" category. Further research is needed to test the effectiveness of ZEO on other postharvest fruit rot fungal pathogens of strawberry, to elucidate the mechanisms of antifungal action of ZEO and to determine if ZEO affects sensory quality of strawberry fruits.

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